Enhancing venom lethality: The incompatibility of *Cannabis sativa* extract in snakebite management

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Running title: Effect of *Cannabis sativa* on cobra venom

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ABSTRACT

Snakebite envenoming is recognized by the World Health Organization (WHO) as a neglected public health issue, particularly in tropical and subtropical regions. Since it has been found that some victims of snake bites use *Cannabis sativa* to fight snakebite envenoming, the objective of this study was to examine the impact of this herb on the lethal effects of *Naja naja*

oxiana cobra venom in mice. This study utilized four protocols and 80 mice in ten equal groups. The intraperitoneal (IP) route was used for injection. In protocol I as control, Group A received 2 mg/kg of venom. Groups B1, B2, and B3 received *C. sativa* extract at 80, 120, and 160 mg/kg doses, respectively. In protocol II, Groups C1 and C2 received simultaneous administration of 80 and 120 mg/kg of the extract, laterally with 2 mg/kg of venom. In protocol III, groups D1 and D2 were injected with 2 mg/kg of venom, followed by the administration of 80 and 120 mg/kg of the extract after a 20-minute interval. In protocol IV, groups E1 and E2 received the pre-incubated (20 minutes) of venom-extract at the similar doses. On average, animals succumbed to death 35 minutes after being injected with venom. The extract significantly reduced this time in groups C1, C2, D2, E1 ($P<0.01$), and E2 ($P<0.001$) compared to group A. The *Cannabis sativa* not only unable to neutralize the lethal effect of *N. n. oxiana* venom, but it also potentiates its effect and significantly decreased the time of the animal's death.

Keywords: Medicinal plants, *Cannabis sativa*, *Naja naja oxiana*, Snakebite

Introduction

For centuries, medicinal plants have been utilized to treat snakebites, particularly in areas with limited access to healthcare. In such cases, traditional herbal remedies may represent the sole option for providing care to victims and preserving their lives (1). Snakebite envenoming is recognized by the World Health Organization (WHO) as a neglected public health issue, particularly in tropical and subtropical regions. According to the WHO, over 5000,000 victims are bitten by venomous snakes annually, resulting in the death of more than 135,000 individuals and leaving more than three times this number disabled (2).

Iran is a temperate region that is the land of 81 species of snakes, 25 of which are venomous and medically significant due to their potential to cause mortality and morbidity (3). A ten-year survey conducted between 2002 and 2011 in Iran documented 53,787 cases snakebite, with 67 resulting in fatalities (4).

The Iranian snake *N. n. oxiana*, (Fig. 1), belongs to the Elapidae family and mainly inhabits the northeastern region of Iran (5). *N. n. oxiana* is a highly venomous snake that possesses a potent neurotoxin with the lowest level of median lethal dose (LD50), making it deadlier than other venomous cobras. It has been reported that its median lethal dose (LD 50), is about 10 μg/mouse

when injected intravenously and verified after 24 hours (6). Its neurotoxic venom has pre- and post-synaptic blocking properties that can paralyze the neuromuscular junction and kill its prey (7).

Fig 1

The clinical symptoms resulting from a bite by *N. n. oxiana* include intense localized effects like pain, redness, swelling, bleeding, blistering, and tissue necrosis that may manifest at the site of the bite. Additionally, neurological complications can arise, including weakness, ataxia, peripheral nerve paralysis, ptosis of eyelids with mydriasis, shock, excessive salivation (sorrhea), and ultimately respiratory arrest (asphyxia) because of respiratory muscles paralysis. These complications have the potential to be fatal (8). Thus, treatment of patients bitten by this cobra requires urgent medical attention.

For over a century, the conventional and effective treatment for envenomation has been the administration of antivenoms soon after a bite. The currently available antivenoms consist of heterologous allergenic proteins, which pose a risk of acute adverse effects, such as potentially life-threatening anaphylactic or pyrogenic reactions. These reactions are usually noticed during the initial hour following administration. Additionally, delayed

reactions of the serum sickness type have also been reported (9). In clinical practice, certain patients may encounter early side effects following antivenom therapy, which can include symptoms such as urticaria, itching, rapid heartbeat, abdominal cramps, nausea, vomiting, bronchospasm, low blood pressure, and angioedema after receiving antivenom therapy (10).

In general, there are several disadvantages associated with antivenoms, including limitations in access, costly production, difficulties in affordability, variation in efficacy, possible low quality, and the use of high and potentially unsafe doses (11). Therefore, it is crucial to continue scientific investigations to develop new neutralizing agents with lower side effects than current antivenoms.

Herbal medicines serve as a great source of numerous pharmacologically active materials and have been utilized for years to treat many medical problems and diseases. Pharmacological studies have demonstrated that a significant number of herbal compounds can counteract the effects of various venoms and toxins (12).

One such herbal extract is *Cannabis sativa*, also known as marijuana, hemp, or ganja, from the Cannabinaceae family. It is an important psychoactive substance that originated in Central Asia (13). *Cannabis sativa*,

has been used for more than 5,000 years to treat various diseases such as hysteria, psychosis, insomnia, nausea, glaucoma, pain, convulsion, inflammation, depression, and as an insect repellent (14). *Cannabis sativa* contains over 538 chemically active compounds, including cannabinoids, terpenoids, flavonoids, ketones, esters, alkaloids, and other phenolic compounds. Also, research suggests that in certain inflammatory conditions, cannabidiol derivative may act as an anti-inflammatory agent similar to dexamethasone (15).

Additionally, it consists of over 140 phytocannabinoids, which exhibit diverse biological and pharmacological activities and have the ability to act on multiple targets (16). Among these phytocannabinoids, the most active component to produce psychoactive effect is trans-delta-9-tetrahydrocannabinol (D9-THC) (17).

Cannabinoids interact with two receptors, namely the type-1 and type-2 (CB1 & CB2) cannabinoid receptors. They also have two endogenous ligands, arachidonoylethanolamine and 2-arachidonylglycerol (18). These cannabinoids have the ability to penetrate the blood-brain barrier (BBB) and attach to their receptors in the central nervous system, leading to psychoactive

effects. This interaction could potentially neutralize or reduce the lethality of the venom, as well as moderate the clinical symptoms and shock caused by a snake bite. Given the impact of *Cannabis sativa* on the nervous system, we hypothesized that its components might interact with the neurotoxic venom of *N. n. oxiana*, which also targets the nervous system. This interaction could potentially neutralize or reduce the lethality of the venom, as well as moderate the clinical symptoms and shock caused by a snake bite.

Furthermore, our research revealed that individuals may use *Cannabis sativa* for a variety of purposes, such as pain relief, stress management, and for the treatment of venomous animals' stings or bites, due to its anti-inflammatory and analgesic effects. Therefore, this study aimed to assess the potential antagonistic effects of *Cannabis sativa* on the lethal activity of cobra snake (*Naja n. oxiana*) venom in mice, addressing the lack of systematic research into the plant's properties as an antivenom.

Materials and Methods

Venom

The lyophilized crude venom of *N. n. oxiana* was kindly supplied by the Razi Vaccine and Serum Research Institute located in Karaj, Iran. It was

stored at 4ºC and was freshly reconstituted in a sterile saline solution just before being administered via intraperitoneal (IP) injection into the mice.

Plant material and extraction of Cannabis sativa

Fresh female *Cannabis sativa* plants were harvested from an agricultural field in the South Khorasan region of Iran, specifically in Se Ghale city (33° 40′ E and 58° 23′ N). The plant specimen was identified as *Cannabis sativa* at the Ferdowsi University of Mashhad Herbarium (13613-FUMH). The collected plant materials were thoroughly washed and then suspended upside down in a dark environment, maintaining a temperature of 28±4°C for a duration of two weeks to facilitate air-drying. Following this, the flowers were detached, chopped into small pieces, and ground into a fine powder.

The extraction process for *Cannabis sativa* was conducted at the Department of Pharmacognosy, Ferdowsi University of Mashhad's Pharmacy College. A total of 20 grams of the powder was mixed with 200 ml of 70% ethanol. The mixture was stirred for one hour at room temperature three times over the course of 48 hours. It was then filtered three times using Whatman filter paper no. 1. The resulting solution was stored in an aluminum-covered glass container to protect it from light. The ethanol was subsequently removed

from the solution using a vacuum rotary evaporator set at 50 °C (IKARV 10, Germany). The obtained solution was then covered and left under the hood until it turned into a highly viscous solution.

Preparation of injectable extract solution

A very thin layer of the extract was spread onto aluminum foil, carefully weighed, and placed in an oven at 60°C to dry completely. Once its weight remained constant, it was considered a dry extract. The injectable solution was prepared by dissolving a proper quantity of *Cannabis sativa* extract in ethanol. Then, Tween 20 (Sigma-Aldrich) solvent was added the mixture, which was vortexed and allowed to evaporate the ethanol. Finally, normal saline was added to the solution at a ratio of 1:1:8. The mixture was vortexed until the extract completely dissolved (19).

Animals

A total of eighty (80) albino mice of both sexes, each weighing between 25-40 g and aged 8-10 weeks, were acquired from the Animal House at Mashhad University of Medical Sciences for this research. The mice were kept in the animal facility of the Faculty of Veterinary Medicine under standard conditions, including a temperature of 24±2ºC, relative humidity of 55±10%, and a 12-hour light/dark cycle, in standard rodent cages. They were given

mouse pellets for nourishment and had access to water at all times. The experimental procedures adhered to the guidelines established by the Animal Ethics Committee at the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, under the approval code (IR.UM.REC.1401.171).

Experimental Protocols

The study examined the efficacy of Cannabis sativa extract in counteracting the lethal effects of *N. n. oxiana* venom through four distinct protocols (I, II, III, and IV), as detailed in Table 1. The animals were divided into ten equal groups as A, B1, B2, B3, C1, C2, D1, D2, E1, and E2. In protocol I, group A, served as the control and treated with 2 mg/kg of *N. n. oxiana* venom. Groups B1, B2 and B3, received *Cannabis sativa* extract at doses of 80 and, 120 and 160 mg/kg, respectively. In protocol II, Groups C1 and C2 were treated simultaneously with 80 and, 120 mg/kg of *Cannabis sativa* extract respectively, and venom at 2 mg/kg. In protocol III, groups D1 and D2 were administered *N. n. oxiana* venom at a dose of 2 mg/kg, and 20 minutes later, they were treated with 80 and 120 mg/kg of *Cannabis sativa* extract, respectively. In protocol IV, the venom was mixed with *Cannabis sativa* extract and preincubated for 20 min at room temperature (26±2°C) prior to injection into animals. Groups E1 and E2 were treated with this mixture at 2

mg/kg of venom and 80 and 120 mg/kg of *Cannabis sativa* extract respectively. The route of administration was intraperitoneal (IP) injection. The duration of survival (in minutes) for each animal following the injection of venom, extract, and venom/extract was documented and statistically analyzed against control groups.

Statistical analysis

The data are expressed as mean ± SEM, and all results were analyzed with SPSS-22 (SPSS Inc., Chicago, Illinois). A one-way analysis of variance (ANOVA) was conducted, followed by post-hoc analyses using the Tukey test. A significance level of $P < 0.05$ was deemed statistically significant.

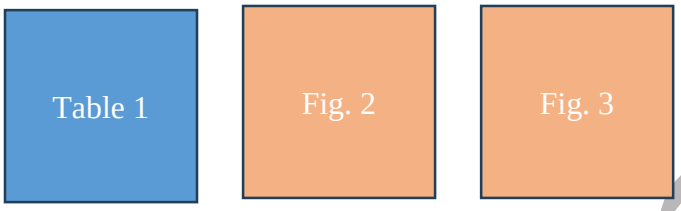
Results

Evaluation the antivenom activity of Cannabis sativa extract

Protocol I, study the acute toxicity

The *N. n. oxiana* venom and *Cannabis sativa* extract was tested in vivo to evaluate their toxic effects. All mice in group A were administered a dose of 2 mg/kg of venom alone. This group exhibited a 100% mortality rate, with an average time to death of 35 minutes (Fig. 2, 3). In contrast, all mice in groups B1, B2, and B3, which received only the *Cannabis* extract at doses of 80, 120,

and 160 mg/kg, respectively, survived, demonstrating that the extract had no toxic effects at the concentrations tested (Table 1).



Protocol II, effect of simultaneous injection of Cannabis sativa extract and N. n. oxiana venom

All mice in Groups C1 and C2 were treated with 80 and 120 mg/kg of *Cannabis sativa* extract, respectively, along with 2 mg/kg of venom simultaneously. In these groups exhibited a 100% mortality rate, with an average time to death of 21 and 17 minutes respectively.

The time to death in animals from Group A showed a significant difference compared to these values ($p<0.001$) (Fig. 2 and 3) (Table 1).

Protocol III, effect of Cannabis sativa extract injected 20 minutes after N. n. oxiana venom

Animals in Groups D1 and D2 treated with 80 and 120 mg/kg of *Cannabis sativa* extract, respectively, 20 minutes after received 2 mg/kg of venom.

In Group D1, the average time to death was 44 minutes, while in Group D2, it was reduced to 23 minutes, showing a significant difference compared to Group A's time to death ($p<0.001$) (Fig. 2, 3) (Table 1).

Protocol IV, effect of mixture of Cannabis sativa extract with N. n. oxiana venom

Group E1 received a mixture of 2 mg/kg of venom and 80 mg/kg of *Cannabis sativa* extract, while group E2 was administered the same dose of venom but with an increased dose of *Cannabis sativa* extract at 120 mg/kg. In group E1, the average time until death was 30 minutes, whereas in group E2, this time decreased to 21 minutes. This difference was statistically significant compared to the time to death for animals in group A ($p < 0.01$ and $p < 0.001$), respectively (Fig. 2, 3) (Table 1).

Discussion

According to the available literature, this is the first assessment of the effects of *Cannabis sativa* extract on the fatality effects of snake venom. Although a dose of 2 mg/kg of *N. n. oxiana* venom resulted in the death of all the mice that were tested, the administration of the extract by itself at doses of 80, 120, and 160 mg/kg showed no toxic effects on the mice. The study

revealed that the *Cannabis sativa* extract not only failed to prevent the death of envenomed mice but also accelerated their time of death. Therefore, the *Cannabis sativa* extract appeared to enhance the fatality effect of the venom of *N. n. oxiana*. The potentiation effect of the extract seemed to be dose-dependent, as the higher dose (120 mg/kg) exhibited a stronger effect than the lower dose (80 mg/kg). The average time of death for animals in all three protocols was 20 minutes at 120 mg/kg, compared to 32 minutes at 80 mg/kg. The shortest time to death (17 min) was observed in protocol II, where the extract was injected at higher doses of 120 mg/kg simultaneously with the venom (Fig. 2, 3). On the other hand, the longest time until animal death was 44 min in protocol III at a dose of 80 mg/kg, which was significantly different ($p<0.001$). The survival time of animals at higher doses of *Cannabis sativa* extract (120 mg/kg) in protocols III and IV was 23 and 21 min, respectively, which is not significantly different. In protocol III, the extract was injected 20 min after venom administration, while in protocol IV, the venom was pre-incubated with *Cannabis sativa* extract for 20 min prior to being administered to the animals. Therefore, these findings suggest that *Cannabis sativa* extract does not physically interact with the venom.

Earlier research has demonstrated that *Cannabis sativa* can penetrate the blood-brain barrier (BBB). Therefore, it is possible that it somehow assists *N. n. oxiana* neurotoxin molecules in passing through cell membranes and reaching their targets faster when injected simultaneously with the venom. In contrast, animals subjected to protocol III exhibited the longest time to death (44 min), where *Cannabis sativa* extract was injected 20 min after venom injection at a lower dose of 80 mg/kg. This time delay may reduce the opportunity for the extract to exert its potentiating effect. However, no direct evidence is currently available for these hypotheses, and further investigations are needed to examine them. While the current study does not provide direct evidence for the mechanisms that explain the significant effects of *Cannabis sativa*, several hypotheses can be proposed based on the existing literature. It has been shown that components of *Naja n. oxiana* venom, such as presynaptic and postsynaptic neurotoxins, can block neuromuscular junctions in different parts of the body.

This occurs either by preventing the release of acetylcholine (Ach) from the nerve terminal or by attaching to the nicotinic acetylcholine receptor (nAChR) particularly in the respiratory muscles, preventing them from twitching and ultimately leading to a lethal effect (20). It has been shown that Cannabidiol

(CBD) exerts pharmacological effects through various specific molecular targets, including nAChR. Therefore, the blocking of nAChR may contribute to the blocking action of *Naja n. oxiana* venom, which is consistent with the findings of this study. Furthermore, several non-psychoactive phytocannabinoids found in *Cannabis sativa*, such as cannabidiol (CBD), cannabidivarin (CBDV), cannabigerol (CBG), and Δ9-tetrahydrocannabivarin (THCV), have been demonstrated to reduce acetylcholine-induced bladder contractions by directly activating smooth muscles in mouse bladders (by blocking muscarinic receptors) (21). Moreover, CBD and CBG have been found to induce the same effect on the human bladder, but not through the cannabinoid CB1 or CB2 receptor mechanisms (22). Additionally, under similar experimental settings, Cannabichromene (CBC) has also demonstrated the ability to inhibit intestinal contractility by reducing electrically-evoked contractions rather than ACh-induced contractions (23). Therefore, it can be hypothesized that the *Cannabis sativa* effects are mediated by its ability to block neuromuscular junctions in mice, potentially enhancing the blocking effect of *N. n. oxiana* venom (21). Further studies, such as using the chick biventer cervicis nerve-muscle preparation during the application of venom and *Cannabis sativa* extract, may provide a more accurate measure to explain

the mechanism of action of this plant. Another hypothesis is that phytocannabinoids and other molecules present in *Cannabis sativa*, especially cannabidiol, potently inhibit many CYP450 enzymes, which alter the metabolism of other drugs and cause their toxicity (24). Therefore, we can assume that *Cannabis sativa* may reduce venom metabolism and elimination by inhibiting CYP or other enzymes responsible for the metabolism of *N. n. oxiana* venom, thereby increasing its bioavailability in the target sites and potentially increasing its toxicity.

However, a lack of basic investigations on the pharmacokinetics of *N. n. oxiana* venom renders these hypotheses unproven, and they require detailed investigation. Additionally, it has been reported that cannabinoids have various short-term effects, including antispasmodic properties and muscle relaxation effects in both human patients with multiple sclerosis (25) and animal models . These effects may also facilitate the paralysis induced by *N. n. oxiana* venom and accelerate the time of animal death, aligning with the results of the present study.

Conclusion

In conclusion, the extract of *Cannabis sativa* does not exhibit any protective or inhibitory effects against the lethality of *Naja n. oxiana* venom in mice. Although the tested concentrations of *Cannabis sativa* extract did not show any toxic effects, they did enhance the lethal effect of *Naja n. oxiana* venom or somehow increase the susceptibility of animals to this venom.

Therefore, *Cannabis sativa* cannot be considered an alternative to conventional antivenoms for treating cobra envenomation, nor can it be used to manage severe pain, intense fear, shock, and anxiety associated with snakebites. In fact, due to its potential to increase the lethality of venom, it is advisable to avoid using this remedy to create a sense of comfort after a snakebite.

Further studies are required to extend these findings to bites from other venomous animals, as well as to isolate the potentiating components and understand their mechanisms of action.

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generously provided the venom. The authors thanks Mrs Monir Taheri for her technical assistance.

Ethics approval

This study was performed according to international code of ethics and approved by the Animal Ethics Committee at the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, under the approval code (IR.UM.REC.1401.171).

Author's contribution statement

B. F. was the principal investigator on this study and was responsible for conducting the design, data collection, writing, and editing of the manuscript. F. S. contributed to the practical work and statistical data analysis. T. Z. contributed to the practical work and data collection.

Conflict of Interest

The authors declare that they have no conflict of interest.

Funding information

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Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Data Availability

The data that support the findings of this study are available on request from the corresponding author

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Figure legends:

Fig. 1. Iranian snake *Naja naja oxiana* (2017-Khorasan)

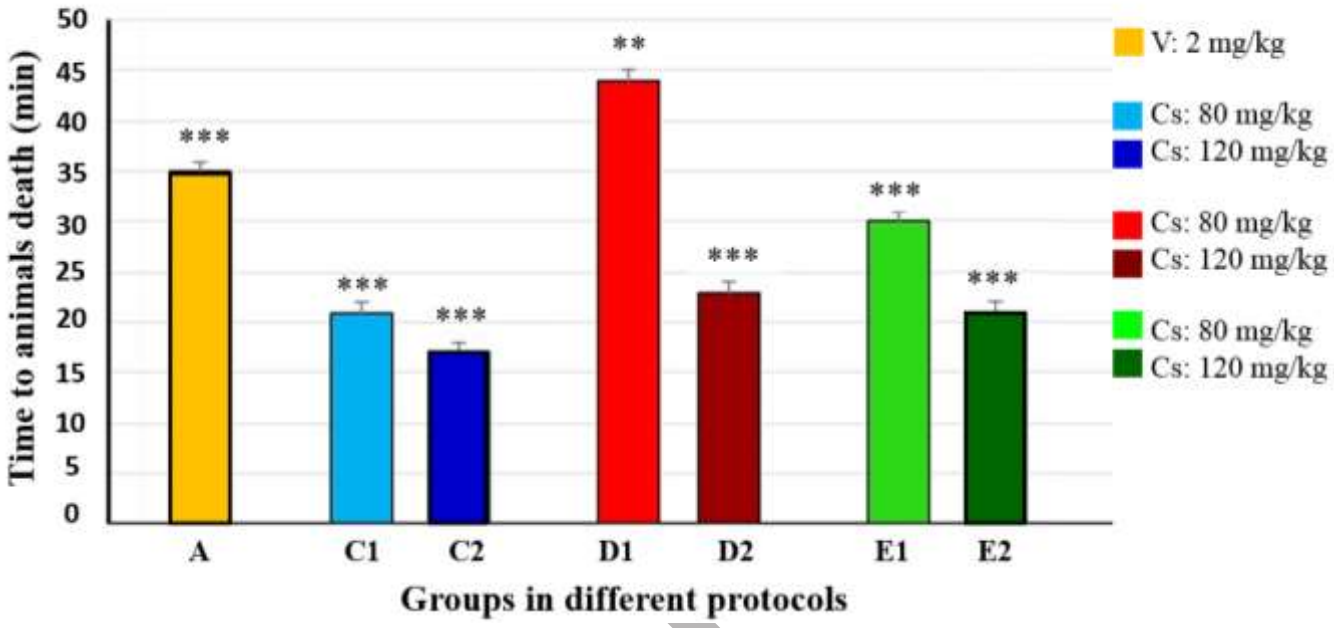
Fig. 2. Time to death of mice after application of venom **(V)** and *Cannabis sativa* **(Cs)** extract in different experimental protocols. Protocols: **I (groups A),** the only venom was injected at 2 mg/kg (control). Protocols: **II (group C1 & C2),** venom and plant extract have been injected simultaneously at 80 and 120 mg/kg respectively, Protocols: **III (groups D1 & D2),** the plant extract has been injected 20 min after to venom injection at pervious doses, Protocols: **IV (groups E1 & E2),** venom and plant extract have been incubated for 20 min prior to being injected at pervious doses. The level of significance considered was $P < 0.05$.

Fig. 3. The comparison chart of death distribution time of animals using a Box Whisker graph in different groups. The bold line in the middle shows the median.

Table 1. Summary of experimental protocols:

Protocol I, (groups A, B1, B2 & B3), the only venom and *Cannabis sativa extract* have been injected (control) respectively. Protocols II, (groups C1 & C2), venom and plant extract have been injected simultaneously, Protocols III, (groups D1 & D2), the plant extract has been injected 20 min after to venom injection, Protocols IV, (groups E1 & E2), venom and plant extract have been incubated for 20 min prior to being injected.

Fig. 1. Iranian snake Naja naja oxiana (2017-Khorasan)



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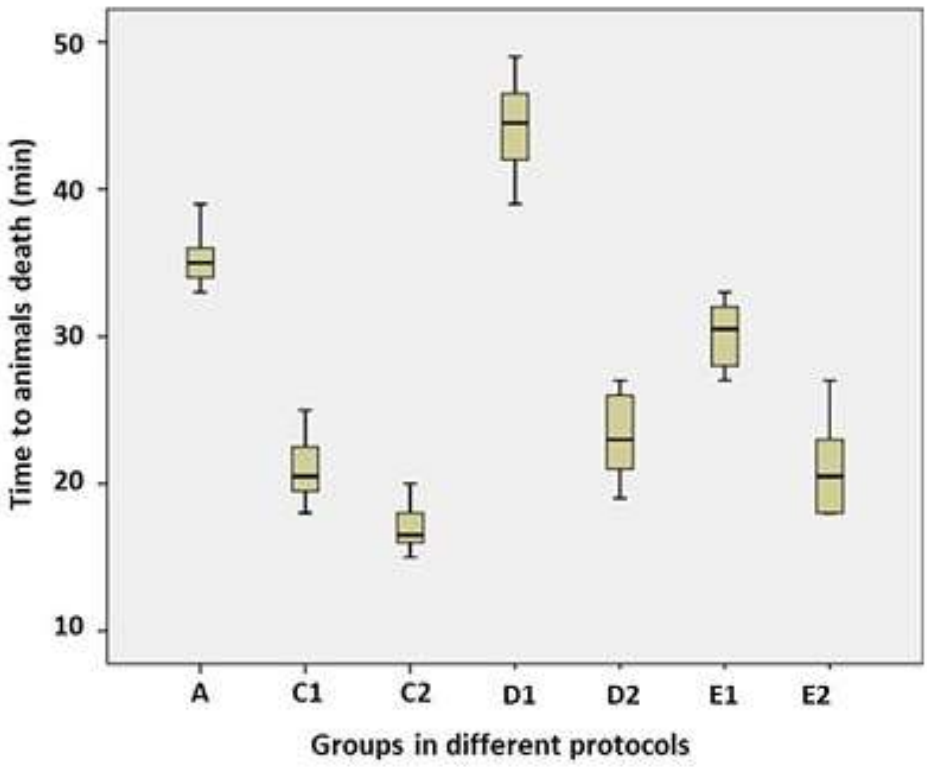

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Protocols	**Groups**	**Number of Mice/Group**	***N. n. Oxiana* venom mg/kg**	***Cannabis sativa* mg/kg**	**Average time to death min**
I	**A**	**8**	**2**	**-**	**35**
I	**B1**	**8**	**-**	**80**	**live**
I	**B2**	**8**	**-**	**120**	**live**
I	**B3**	**8**	**-**	**160**	**live**
II	**C1**	**8**	**2**	**80**	**21**
II	**C2**	**8**	**2**	**120**	**17**
III	**D1**	**8**	**2**	**80**	**44**
III	**D2**	**8**	**2**	**120**	**23**
IV	**E1**	**8**	**2**	**80**	**30**
IV	**E2**	**8**	**2**	**120**	**21**